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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

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Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

96201766.1

PRIORITY DOCUMENT

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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Blatt 2 der Bescheinigung
Sheet 2 of the certificate
Page 2 de l'attestation

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Bezeichnung der Erfindung: Title of the invention: Titre de l'invention:

Vaccine comprising antigens bound to carriers through labile bonds

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The original title of the application reads as follows :
"Immunogenicity increasing labile linking of antigens to carrier compounts".

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Title: Immunogenicity increasing labile linking of antigens to carrier compounds.

The invention relates to the field of immunization and vaccines. By vaccination, antibody responses are elicited in the vaccinated animal or human. These antibody responses are directed against the antigen or antigens that are used in the vaccine. It has long been known that the antigenicity and immunogenicity of antigens may vary, depended for example on the way the antigen is presented to the vaccinated animal, or on the size and structure of the antigen molecule.

A widely known way of increasing the immunogenicity of a vaccine preparation is the addition of adjuvants to this preparation. However, such adjuvants stimulate the immune response only in an aspecific way, and do not increase the specific antigenicity or immunogenicity of the antigen used.

Enhancement of specific antigenicity or immunogenicity of antigens that are themselves poorly antigenic or immunogenic is often achieved by coupling the antigen to carrier compounds. Synthetic peptides have frequently been used to immunize animals. Peptides of less then about 20 to 30 amino acids are, however, known examples of such poor immunogens. To increase their specific antigenicity or immunogenicity it is assumed that the molecular weight needs to be increased.

Many attempts have been made to conjugate antigens in a stable bond to carrier molecules, using different carrier compounds such as the carrier proteins keyhole limpet haemocyanin (KLH) or ovalbumin (OVA), in order to increase the molecular weight. Such immunoconjugation may lead to very complex structures, and generate unwanted side-effects, in the sence that unwanted antibodies are elicited that are specifically directed against the carrier compound and the areas of the conjugated molecule where the linkage of the antigen with the carrier have taken place.

Other methods that have been applied are the coupling of fatty acid groups to proteins or peptides of low antigenicity or immunogenicity. The main purpose of the introduction of fatty acids into antigens has been to anchor hydrophillic antigens on adjuvant- and immuno-presentation systems, e.g. liposomes and iscoms, because such systems require the presence of hydrophobic molecules to allow their incorporation into these systems. Such procedures of vaccine preparation have been used with some success with model protein antigens and with less success with synthetic peptides. Acylation of the lysines of ovalbumine with palmitic acid was found to enhance the major histocompatibility complex (MHC) class II-restricted presentation. This suggests that conjugation with longer fatty acids would lead to the formation of lipopeptide T-cell epitopes with increased affinity for binding to MHC class II and/or T-cell receptors (20).

Such coupling of fatty acids has been achieved by acylation of proteins or peptides (26) intended for use in immunization/vaccination purposes, simply by linking the fatty acid covalently to the antigen. In these studies, predominantly palmitic acid or myristic acid have been used. For protein antigens, the introduction of 'lipid tails' (palmitylation) has been performed mainly by using palmitic acid N-hydroxy succinimide, leading to an irreversible and stable bond between the protein and the fatty acid. For synthetic peptide antigens, the most common procedure is performed after synthesis of the peptide, by adding palmitic acid to the free amino group in the terminal amino acid by continuing peptide synthesis chemistry. This leads to an irreversible amide bond between the palmitic tail and the peptide.

We have now found a procedure which, instead of coupling the antigen irreversibly to a carrier compound, allows for coupling between the antigen (be it protein or peptide or carbohydrate or any other molecule to be used as an antigen for immunization/vaccination procedures) and the

carrier compound in a reversible and labile way, with a so-called labile-linking method. With a 'labile-link' a labile chemical bond between antigen and carrier protein is meant. Labile should be understood as either chemically or enzymatically labile. A chemically labile bond cleaves for example under conditions normally found in the body, i.e. at basic pH such as can be found in body tissue, whereas an enzymatically labile bond cleaves in the presence of enzymes, such as thioesterases or esterases, which are present in bodily tissues.

Such cleavages result in a dissociation of the antigen and the carrier compound, after administration of the vaccine. In this way, as will be demonstrated in detail in the experimental part of this description, surprisingly a better immune response can be elicited by an in itself poorly immunogenic antigen than by methods that provide a stable link between the antigen and carrier compound.

The invention not only allows for the introduction of carrier compounds, such as lipid tails, into antigens but also allows for a better routing to the cell surface of antigen presenting cells after which the antigen becomes dissociated and may be better processed by those antigen presenting cells, resulting in a much better immune response. Therefore, the invention is not simply aimed at anchoring antigens to carrier proteins, adjuvants, or other presentation systems, but is in its own right capable of greatly increasing the antigenicity or immunogenicity of antigens.

A theoretical explanation which should not be seen as restricting the invention is that antigens with reversible bonds or labile links to carrier compounds, for instance by palmitic acid acylation of thiol groups in peptides, would facilitate the routing of these antigens to various cell compartiments and enhance in this way their immunogenicity.

Peptides have been substituted with palmitic acid residues in quite a number of ways: by conjugation with Nα,NE-dipalmitoyllysine peptides (8,5), with Na-palmitoyl-S-

(2,3-*bis*-palmitoloxy-(2RS)-propyl)-(R)-cysteine (Pam_3Cys-peptides (5,13)), palmitoylated polylysine (23,24), Pam_3Cys-Multiple Antigen Peptides (4,10), lipo-Multiple Antigen Peptides (9,10), N-palmitoylated proteins used for incorporation into ISCOMs (3,14,17), and peptidyl-NÓ-palmitoyl-lysines (5). In these examples, palmitoylation was at an amino group or a hydroxyl group, providing irreversible bonds such as amides.

The present invention as exemplified in the experimental part, which utilizes the formation of a labile bond, now provides a very simple approach to obtain labily linked antigen and carrier compound via the controlled conjugation of the antigen with one long-chain fatty acid. The presence of the fatty acids increases the uptake of the antigen into antigen presenting cells, and, due to the labile or reversible nature of the bond, the antigen becomes separated again, and specific antibody formation directed to the antigen only is elicited.

In the experimental part the concept of labile linking is demonstrated with synthetic peptides as antigen linked via a thioester bond to palmitic acid as carrier compound. A preferred part of the invention is thus illustrated by the examples provided. However, any vaccine or immunogenic preparation in wich the antigen is reversibly or instabily or labily bound to the carrier compound by whatever labile link imaginable with the purpose that the antigen dissociates from the carrier compound after administration of the vaccine is also part of the invention. For example, a vaccine composition comprising a glycopeptide, linked via a disulfide bridge to ovalbumine, will dissociate as soon as the vaccine composition is met with reducing circumstances, as for instance can occur in the blood in the presence of glutathion.

As antigen, other types of molecules to which antibodies preferably need to be elicited, such as polypeptides, proteins with or without carbohydrate moieties or other side chains, carbohydrate chains themselves,

haptens, etc., can be chosen. As carrier compound, other fatty acids can be used, but also carrier proteins, such as KLH or OVA, or other types of molecules can be used as carrier compound.

For the labile link between the antigen and the carrier compound, the thioester bond illustrates a prefered part of the invention. However, other chemical bonds such as esters and disulfide bonds that are labile under physiological conditions (such as pH or osmolarity or salt concentrations or the presence of reducing agents or enzymes) which are usually present in body fluids or in or on body tissue or cells also fit the purpose of providing a vaccine or immunogenic preparation in which the antigen dissociates from its carrier compound under physiological conditions, i.e. after it has been administered.

Also, vaccines and immunogenic preparations that are prepared according to the invention can be mixed with carrier compounds and adjuvants that are traditionally used to render antigens more antigenic or immunogenic. In addition, vaccines and immunogenic preparations that are prepared according to the invention can be mixed with other compounds or adjuvants or antigen delivery systems that are specifically chosen to hasten or slow the rate of dissociation of the antigen from the carrier compound after the preparation has been administered, i.e. by surrounding the preparation with conditions in which the dissociation rate is altered, and thereby shielding it temporarily from normal physiological conditions.

Furthermore, vaccines and immunogenic preparations that are prepared according to the invention can be administered in various ways known in the art, for instance by but not limited to intramuscular, intranasal, intraperitoneal, intradermal, intracutanous, mucosal or aerosol application of the preparation.

EXAMPLES

Introduction

The concept of labile linking is demonstrated with synthetic peptides as antigen and palmitic acid as carrier compound.

Conditions for acylation of peptides in solution or on solid phase were established. Furthermore, it was demonstrated that the stability of the bond influences the immunological efficacy, because in our study conjugation of a fatty acid implies either formation of a stable amide when N-acylation is involved, or formation of a labile thioester in the case of S-acylation. It was found that this difference in site of acylation (N or S) is important with respect to its immunogenicity. Upon immunization it was found that S-palmitoylated peptides are superior to N-palmitoylated peptides and at least comparable to KLH conjugated peptides with respect to response time of detectable antibody formation or biological effect. A theoretical explanation is that, under biological or physiological conditions, the presence of appropriate fatty acid chains chemically linked through labile thioester bonds, improves immunogenicity, probably because it represents a favourable substrate for take up and processing in cells of the immune system.

We investigated S-palmitoylation of peptides to test the hypothesis whether there would be any immunogenic advantage in comparison to N-palmitoylation. Unlike amides and esters, thioesters are readily susceptible to nucleophilic attack and palmitoyl thioesterases have been isolated (18). The chemical stability of palmitoyl thioesters had thus to be considered at physiological pH and during deprotection conditions (acid, acid-thiol, base). Seven peptide constructs (A, B, C, D, E, F and G, Fig. 1) were prepared as vaccine preparation, two peptides represent a GnRH-tandem peptide and five are based on the N-terminus of VP2 (positions cys-2-21) of canine parvovirus (CPV (11)).

They differ by their acylation sites (S, α-N or E-N, Fig. 1). It was found that S-palmitoylated peptides are comparable to or slightly better than KLH-MBS linked peptides, and superior to N-palmitoylated peptides with respect to response time of detectable antibodies (CPV) or biological effect (GnRH).

MATERIALS:

N-methylpyrrolidone (NMP), 2-(1H-benzotriazol-1-yl)-1,1,3,3,-tetramethyluronium hexafluorophosphate (HBTU) and piperidine were peptide synthesis grade and obtained from Perkin Elmer/ABI (Warrington, UK). Dicyclohexylcarbodiimide (DCC), dimethylformamide (DMF), N-hydroxybenzotriazole (HOBt), diisopropylethylamine (DIEA), acetonitril (ACN), trifluoroacetic acid (TFA), thioanisole (TA), phenol, palmitic acid and ethanedithiol (EDT) were pro-analysis grade and were obtained from Merck (Darmstadt, Germany). Diethylether was purified over a column of activated basic aluminumoxide and DIEA was distilled twice over ninhydrin and potassiumhydroxide before use. Fmoc-amino acid derivatives and resin (4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy resin (Rink resin) (19)) were obtained from Saxon Biochemicals (Hannover, Germany). 4-methylbenzhydrylamine (MBHA) resin was obtained from Novabiochem, Laufelfingen, Switzerland.
For analytical HPLC we used two Waters pumps model 510, a Waters gradient controller model 680, a Waters WISP 712 autoinjector, and a Waters 991 photodiode array detector. Products were analyzed in a linear gradient from water with 0.1% TFA to 60% acetonitril/water with 0.1% TFA in 60 minutes on a Waters Delta Pak C_{18}-100 (3.9x150mm, 5Êm) column at 1 ml/min.

Amino acid analysis was performed using a Waters Pico-Tag system, after hydrolysis in a Pico-Tag workstation using 6N HCl at 150°C for 1 hour, and derivatization with phenylisothiocyanate.

Preparative HPLC was carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cardriges plus guard cardridge (40x210 mm or 25x210 mm) filled with Delta-Pak C_{18}-100 (15 Êm) material. Peptides were detected at 230 nm using a Waters 486 spectrophotometer with a preparative cell.

METHODS:

1) Synthesis of peptides.

All peptides (except compound B: Fig. 1) were synthesized as amides on a Rink resin (loading 0.38 mmol/g), using the FastMoc™ method (7) on an ABI 430A peptide synthesizer. Cysteine sulphur was protected with a S-tert-butylsulfenyl group or a trityl group, all other side chain functions were protected with trifluoroacetic acid labile groups. After completion of the synthesis, peptides were either acetylated using acetic anhydride/DIEA/NMP (0.1/0.01/1 (v/v/v)) or N-palmitoylated using pre-activated palmitic acid (see Methods; sections 3 and 4). Peptides with S-trityl protected cysteine were cleaved from the resin and concomitantly deprotected by acidolysis in the presence of scavengers using TFA/water/EDT/TA/phenol (40/2/1/2/3) (v/v/v/v/w)) for 2 hours. The crude peptides were precipitated and washed twice with hexane/ether (1:1) and lyophilized from acetonitril/water (1:1). Compound B was synthesized differently. The synthesis started with acylation of the amino group on a MBHA-resin (loading 0.46 meq/g) with Fmoc-Lys(Boc)-OH in NMP. After cleavage of the Boc group by TFA/water (19:1), lysine was palmitoylated with pre-activated palmitic acid (see Methods; section 4). After Fmoc deprotection the α-amino groups were successively acylated with the pertinent Fmoc-amino acids to give the protected peptide. Compound B was deprotected and cleaved from the resin by HF/anisole (9:1).

2] Deprotection of S-(tert-butylsulfenyl)cysteinyl residues in solution.

The side chain protection of cysteine was removed using a reducing agent: a double deprotection of 30 minutes was routinely used, each with a 50 eq. molar excess tri(n-butyl)phosphine in 10% H_2O/NMP under nitrogen atmosphere.

3] S-palmitoylation in solution.

-*Activation of palmitic acid.* To a solution of palmitic acid (100 µmol) in DCM (250 µl) was added 100 µmol of HBTU and HOBt (0.45M in DMF; 220 µl) and 200 µmol 2M DIEA in NMP. The activated palmitic acid formes a gel from this mixture in about 20 minutes at room temperature.

-*Palmitoylation of a thiol group.* An acetylated peptide (not containing lysyl residues) was dissolved in NMP at a concentration of 10 mg/ml. The solution was brought to pH 5 with DIEA (2M in NMP), as checked by using wet pH paper, and mixed with one eq. of pre-activated palmitic acid. After 90 min at room temperature and stirring, a sample was subjected to analytical HPLC. About 50% of the peptide was found to have reacted. A second equivalent of pre-activated palmitic acid was added and after another 90 min 70% of the peptide was converted and no by-products were found. After a total reaction period of 5 hours, the mixture was diluted fourfold with acetonitril/water (1:1), two drops of TFA were added and the palmitoylated peptide was isolated and purified by preparative HPLC

-*Palmitoylation of an α- or an E-amino group.* A free Nα or a free NE of a lysyl residue were treated as decribed for a thiol group.

4] S-palmitoylation on the resin.

After completion of the synthesis, the resin was treated twice for 30 minutes with tri(n-butyl)phosphine (50 eq.) in NMP/water (9:1) to remove the tert-butylsulfenyl group. The resin was washed copiously with NMP. Free SH groups were palmitoylated on the resin by double coupling

using the pre-activated palmitic acid reagent (see palmitoylation in solution, section 3) in DCM/NMP for 5, resp. 16 hours. The second time, only 1 eq. DIEA was added.

5] Selection of deprotection mixture and stability of thioester with respect to acid.

To investigate the stability of thioesters during acidolytic removal of tert-butyloxy functions (Boc and OBu^t) and/or cleavage of the resin-peptide bond, two model peptides, Ac-C(palm)SEIFRPGGGDMR-NH_2 and Ac-C(palm)VATQLPASF-NH_2, both not containing lysine, were palmitoylated in solution and purified with preparative HPLC. Samples were then subjected to the conditions of acidolysis in the presence or the absence of scavengers using TFA/water (19/1 v/v) (A), TFA/water/EDT/TA/phenol (40/2/1/2/3 v/v/v/v/w) (B), or EDT/water/TFA (1/1/38 v/v/v/) (C). Acetic acid/water (1/1) was used as a control. The treatment (3 hours) was at room temperature.

6] Blocking of free SH groups by iodoacetamide.

To prevent the peptide ac-CSDGAVQPDGGQPAVRNERTAG-NH_2 from dimerization by air oxidation, the free SH group was blocked. Peptide ac-CSDGAVQPDGGQPAVRNERTAG-NH_2 (20 µmol) and iodoacetamide (100 µmol) were dissolved in 400 µl DMF and 600 µl 2% NH_4HCO_3 (pH 8.0). The pH was kept between 7.5 and 8.5, and if necessary corrected with extra NH_4HCO_3 (grains). The solution was stirred for some 1.5 or 2 hours at room temperature. Acetic acid (100%, 50 µl) was added to stop the reaction. An Ellmann test was used to analyse the result.

7] Stability of the thioester with respect to base.

The stability of peptide A was investigated in aqeous NH_4HCO_3 (2%, pH 8) and in 30% piperidine in NMP. The results are summarized in table 1.

8] Characterization of S-palmitoylated peptides by FAB-MS.

Following purification the molar mass was determined of the palmitoylated CPV peptides C (average mass calc: 2422, found: 2489) and E (average mass calc: 2465.79, found 2465.20). It turned out to be that compound C had an extra piperidyl moiety at one of the aspartic acid residues at position 3 or 9. Since FAB-MS gives no information on the site (N or S) of acylation, mild alkaline hydrolysis of the thioester was used to regain the original peptide; amides are stable under these conditions. The UV spectrum of the S-palmitoylated peptide showed a slight elevated absorption at 254 nm compared to the N-palmitoylated product. Once the thioesters are set free, they tend to undergo oxidative dimerization when exposed to air; addition of (the relative stable)β-mercaptoethanol will prevent this reaction and the peptides can be recuparated *e.g.* for analysis.

9] Conjugate preparation.

Keyhole limpet haemocyanin (KLH, 10 mg, Calbiochem, La Jolla, CA) was dissolved in 0.1 M sodiumphosphate buffer, pH 7.0, at a concentration of 12.5 mg/ml. Acetonitril (0.3 ml) was added, followed by 100 µl of 0.125 M maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, Pierce, Rockford, IL) solution in DMF. After stirring for 1 hour, the solution was dialysed three times for 30 minutes against 0.1 M phosphate buffer, pH 7.0 at 4°C. Peptide (10 mg) was added to the modified KLH and the mixture was shaken overnight at room temperature. The conjugate was dialyzed two times for 2 hours and then overnight against 0.1 M phosphate buffer, pH 7.0 at 4°C.

10] Vaccine formulation.

For each vaccine, peptide constructs (conjugated to KLH, N- or S-palmitoylated) were dissolved in phosphate-buffered saline (PBS, pH 7.2) and emulsified with complete Freund's adjuvant (CFA) in equal volumes. Mixtures were emulsified by repeatedly forcing it through a needle until a

stable water-in-oil emulsion was obtained. The emulsion was prepared just before vaccination. Second vaccinations were prepared with incomplete Freund's adjuvant (IFA).

111 Immunization.

GnRH-tandem constructs. Six groups of 7-8 male piglets at the age of 10 weeks were vaccinated with compound A or B (Fig. 1). Each animal was injected with 2 ml vaccine formulation (1, 0.25 or 0.05 mg) administered intramuscularly in the neck. The second vaccination was given 8 weeks later. One group served as a control and was mock vaccinated with only CFA and KLH. At week 26 the animals reached their slaughterweight; their testicles were excised, epididymes were removed and testes weight was recorded.

CPV constructs. In the first experiment, two groups of 5 guinea pigs were vaccinated with compound C or D (Fig. 1). Each animal was injected with 500 Êl vaccine formulation administered subcutaneously in the neck. The second vaccination was given 6 weeks later. One group served as a control and was vaccinated with compound E (KLH-MBS coupled peptide construct, Fig. 1). Blood was taken at weeks 0, 6, 7 and 12 post vaccination and anti peptide antibody titers were recorded in an ELISA.

In the second experiment, one group of 3 guinea pigs was vaccinated with compound C emulsified in CFA. For comparison, compound E was emulsified in CFA. Blood was taken at 0, 4, 8 and 16 weeks post vaccination and anti peptide antibody titers were recorded in an ELISA.

In the third experiment, three groups of three guinea pigs were vaccinated with compound D, E or F. CFA was used to emulsify the peptides. As a control, the free peptide G was used in which the thiol group was blocked with iodoacetamide to prevent dimerization of the peptide. Blood was taken at 0, 4 and 8 weeks post vaccination and anti peptide antibody titers were recorded in an ELISA.

Results

1] Methodological.

In this investigation a method has been developed for the acylation of peptide thiols in solution or within the matrix of the solid support. In solution, peptides with only one single nucleophilic function (N or S) can be acylated in a very efficient way using pre-activated palmitic acid. The solid support approach requires an efficient and selective removal of the *tert*-butylsulfenyl protection, which was realized effectively only by reduction with tri(n-butyl)phosphine in NMP/water (9/1). An advantage of this approach is that it can be done in the presence of lysines, because the lysines are still protected. The thiol palmitates (A, C and E; Fig. 1) were obtained by acylation with an active ester of palmitic acid in the presence of a base; they were found stable during acidolysis of other protective functions. The palmitoyl-thioester bond is not stable under basic conditions. Buffered solutions of the thioesters around pH 8 should not be left standing for prolonged periods (Table 1).

2] Immunogenic potency of palmitoylated peptides.

a] GnRH-tandem peptides A and B:

The immunogenic potency of the synthetic GnRH tandem peptides A and B was tested in an experiment on six groups of 7-8 male piglets (age 10 weeks), each group recieving a dose of either S-palmitoylated (A) or N-palmitoylated (B) antigen. The GnRH tandem peptides A and B exhibited different immunogenic effects, as observed on testis weight (15) at the age of 26 weeks after two injections in the presence of CFA (see Table 2). While both forms were able to keep testis size low, only the S-palmitoylated peptide A was able to do this in all animals at the highest dose tested (1 mg), and still in 3 out of 8 animals at the lowest dose (0.05 mg) tested. The response was also dose dependent. A

dose of 0.25 mg S-palmitoylated peptide A gave a comparable effect as a dose of 1 mg N-palmitoylated peptide B.

b] CPV peptides:

The above observed effect with the palmitoylated GnRH peptides A and B was further investigated with another epitope, and in another animal species. In following experiments, the N-ternimal fragment of VP2 (positions 2-21) of canine parvovirus was used. This peptide, when S-palmitoylated (peptide C, Fig. 1), appeared to be capable to elicit antibody responses as well as the peptide conjugated to KLH (peptide F, Fig. 1). On the other hand, the N-palmitoylated peptide (peptide D, Fig. 1) did yield a lower immunogenic effect, since antibody titers were lower and did not develop in all animals (see Table 3). According to the FAB-MS results, the S-palmitoylated peptide C had a mass of 67 units higher than expected. This turned out to be a piperidyl moiety, coupled to the side chain of an aspartic acid at position 3 or 9. On the basis of this result, the synthesis of peptide E was adapted. To exclude the immunogenic influence of the extra piperidyl moiety, a *second experiment* was performed and peptides C and E were compared in CFA. It was confirmed that the immunogenicity of the compounds C and E was comparable, and the immunogenicity did not depend on the presence of a piperidyl group, nor the absence of an amino terminal acetyl group (see Table 4). The superiority of the S-palmitoylated product above the N-palmitoylated peptide was further confirmed by direct comparison of peptide D, peptide E, and the KLH-MBS-conjugated peptide F (Table 5). In this *third experiment* the antibody titer elicited with the S-palmitoylated peptide at 4 weeks past vaccination was almost 100 times higher than the titer obtained with the N-palmitoylated peptide. At week 8 the titers were almost equal. Compared to the KLH-conjugated peptide, the titers for the S-palmitoylated peptide were both in the 4th and the 8th week higher.

Discussion of experimental results in the examples

Immunogenic aspects.

In this study, procedures for palmitoylation through an amide or thioester bond were established. Furthermore, we investigated the immunogenic potency of peptides conjugated to a fatty acid, and compared it with KLH-MBS-conjugated peptides. It was found that the site of acylation (N or S) effects the immunogenicity of the adduct. Surprisingly, palmitoylation of the peptide through a thioester bond (S acylation) appeared to yield a highly immunogenic product, being as potent as or even better than a peptide coupled to a carrier protein (KLH or OVA). On the other hand, N-palmitoylation is far less immunogenic compared to S-palmitoylation or KLH-MBS conjugation. The introduction of a fatty acid through a thioester bond thus obviates the necessity of a large carrier protein. The conventional conjugation with a large molecule (a protein) induces difficulties because the chemistry underlying the coupling is not always well controlled, while in addition unwanted antibodies against carrier protein and linker are induced.

Chemical aspects:

Nα-acetylated peptide amides can be prepared automatically through solid-phase synthesis. However, removal of the base-labile Fmoc groups with piperidine precludes the use of S-palmitoylated cysteinyl derivatives during synthesis. Therefore, S-acylation had to be performed at the end of the synthesis on the resin or after cleavage and deprotection in solution. The acylation reaction applied here can not discriminate between thiols and amino groups. Since peptides upon cleavage from their support loose also their N-protecting groups, selective palmitoylation can only be performed in solution with compounds comprising one single nucleophilic function (N or S). Hydroxyl groups are not affected.

Because of the instability with respect to base it is not recommended to leave S-palmitoylated peptides in buffered (pH 7-8) solutions for prolonged periods (see Table 1). Thioesters do not dissociate in acidic media and do thus not contain thiolate anions which would attack thioesters. We confirm the general rule that thioesters resist thiols in strongly acidic media enabling the use of thiols as scavengers for carbonium ions during deprotection of *tert*-butyloxy functions (cf. 21).

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Fig. 1; Peptide sequences used for immunization experiments.

GnRH peptides:

A: /EHWSYGLRPGQHWSYGLRPGC(palm)-NH_2 (thioester)

B: /EHWSYGLRPGQHWSYGLRPGK(palm)-NH_2 (amide)

/E= pyroglutamic acid

CPV peptides:

C: Cys(palm)SDGAVQPDGGQPAVRNERATG-NH_2 (thioester)

D: palmCysSDGAVQPDGGQPAVRNERATG-NH_2 (amide)

E: ac-Cys(palm)SDGAVQPDGGQPAVRNERATG-NH_2 (thioester)

F: ac-Cys(MBS-KLH)SDGAVQPDGGQPAVRNERATG-NH_2 (conjugated)

G: ac-Cys(CH_2-C_O-NH_2)SDGAVQPDGGQPAVRNERATG-NH_2 (blocked)

Table 1. Stability of the thioester bond in basic solutions

	time	
	2 hours	22 hours
2% NH_4HCO_3 (pH 8)	>97%[a]	~30%[a]
30% piperidine/NMP	0%	0%

[a]: percentage of GnRH tandem Cys(palm) peptide A as estimated by HPLC

Table 2: Biological response of male piglets immunized with different doses of GnRH tandem peptides.

peptide	dose (µg)	testis weight[a]		
		<100	100-150	>150
A	1000	7/7	0/7	0/7
A	250	6/8	1/8	1/8
A	50	3/8	0/8	5/8
B	1000	5/7	0/7	2/7
B	250	1/7	2/7	4/7
B	50	0/6	0/6	6/6
control	KLH	0/7	0/7	7/7

[a]: testis weight less then 100 gram (positive), between 100-150 gram (intermediate) and more than 150 gram (negative) (15)

Table 3: Serum anti-peptide antibody titers at 6, 7 and 12 weeks of guinea pigs vaccinated with 100 µg compound C, D or F emulsified in CFA. Booster vaccination emulsified in IFA was given at week 6 post first vaccination. Blood samples were already taken.

		titer[a]		
animal	compound	6wpv	7wpv	12wpv
1	C	5.1	6.0	>6.5
2	C	5.1	5.0	4.8
3	C	6.1	5.4	5.4
4	C	5.0	4.9	5.0
5	C	4.6	#[b]	
6	D	3.5	4.1	4.3
7	D	1.7	1.6	2.9
8	D	3.5	3.7	3.7
9	D	-[c]	-	-
10	D	2.0	2.0	#
11	F	5.1	5.3	5.7
12	F	5.0	5.0	>6.5
13	F	4.5	4.7	#
14	F	5.2	#	
15	F	5.0	5.2	5.3

[a]: zero blood samples were subtracted before calculation. The titer was calculated as the -log(dilution factor) which give a signal three times the background.

[b]: animal died during the experiment.

[c]: means no detectable titer, lowest dilution measured was 1/10

Table 4: Serum anti-peptide antibody titers after 4, 8 and 16 weeks in guinea pigs. Compound C was compared with compound E, both emulsified in CFA.

		titer[a]		
animal	compound	4wpv	8wpv	16wpv
1	C	2.2	3.7	3.8
2	C	2.1	2.4	2.8
3	C	2.2	3.8	3.8
4	E	-[b]	3.0	4.0
5	E	2.1	3.5	4.6
6	E	2.1	2.4	2.5

[a]: zero blood samples were subtracted before calculation. The titer was calculated as the -log(dilution factor) which give a signal three times the background.

[b]: - means titer lower than 2.0

Table 5: Serum anti-peptide antibody titers after 4 and 8 weeks of guinea pigs immunized with 100 µg palmitoylated peptides (S or N), conjugated (KLH-MBS) or blocked (iodoacetamide).

animal	compound	titer[a] 4 wpv	8 wpv
1	E	2.5	2.5
2	E	4.0	3.3
3	E	3.1	3.4
4	D	1.8	2.9
5	D	-[b]	3.4
6	D	-	2.3
7	F	2.5	2.2
8	F	2.7	2.0
9	F	-	-
10	G	-	-
11	G	-	-
12	G	-	-

[a]: zero blood samples were subtracted before calculation of the titer. The titer was calculated as the -log(dilution factor) which give a signal three times the background.

[b]: - no detectable titer, lowest dilution measured was 1/30.

Ren/GW Eur 3981/WvA

CLAIMS

1 A vaccine or immunogenic preparation comprising an antigen and a carrier compound which are linked by a chemical bond that is labile under physiological conditions.

2 A vaccine or immunogenic preparation preferably according to claim 1 in which the antigen dissociates from the carrier compound after the vaccine or preparation has been administered.

3 A vaccine or immunogenic preparation according to claim 1 or 2 in which the antigen can be a protein, a polypeptide, a synthetic peptide, a carbohydrate, or a hapten.

4 A vaccine or immunogenic prparation according to any of claims 1-3 in which the carrier compound is a protein or a fatty acid.

5 A vaccine or immunogenic preparation according to any of claims 1-4 in which the chemical bond is a thioester or a disulphide bond.

6 A vaccine or immunogenic preparation according to claim 1 or 2 in which the antigen is linked by thioester acylation to a fatty acid.

7 A vaccine or immunogenic preparation according to claim 6 in which the antigen is a synthetic peptide.

8 A vaccine or immunogenic preparation according to claims 6 or 7 in which the fatty acid is palmitic acid.

9 A vaccine or immunogenic preparation according to claim 7 or 8 in which the synthetic peptide consists of the amino acid sequence EHWSYGLRPGGHWSYGLRPG.

10 A vaccine or immunogenic preparation according to claim 7 or 8 in which the synthetic peptide consists of the amino acid sequence SDGAVQPDGGLPAVRNERATG.

11 A vaccine or immunogenic preparation according to any of claims 1-10 together with an additional pharmaceutically acceptable compound or adjuvant.

ABSTRACT

The invention is in the field of vaccines and immunogenic preparations. Normally in these preparations antigens and carrier compounds are irreversibly coupled in a stable bond. The invention, to the contrary, provides vaccines and immunogenic preparations in which the antigen (be it protein or peptide or carbohydrate or any other molecule to be used as an antigen for immunization/vaccination procedures) and the carrier compound are coupled in a reversible and labile way, with a so-called labile link. In this way, as is demonstrated in the experimental part of this application, surprisingly a better immune response can be elicited by an in itself poorly immunogenic antigen than by methods that provide a stable link between the antigen and carrier compound.